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*Original Research*

# Assessment of cytotoxic and apoptotic properties of Thymoquinone-oxime derivative on U87 glioma cells

Thymoquinone-oxime derivative

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**Abstract**

Aim: Glioblastoma, characterized by its invasive nature, stands as one of the prevalent primary malignant brain tumors. Thymoquinone (TQ) has demonstrated notable anticancer effects across various cancer types. Nevertheless, the comprehensive molecular underpinnings behind TQ's anticancer efficacy remain partially elucidated. Given TQ's capacity to traverse the blood-brain barrier and its selective cytotoxicity towards glioblastoma cells over primary astrocytes, it emerges as a highly promising chemotherapeutic agent for gliomas and glioblastomas. This study's principal objective was to delve into the impact of Thymoquinone-Oxime (Ox) on cytotoxicity and apoptotic attributes within the U87 cell line.

Material and Methods: Various concentrations of TQ-Ox (1.56-200 µM) were applied to the U87 glioma cell line for a 24-hour incubation period. Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while apoptosis quantification utilized Annexin V and7-aminoactinomycin D (7-AAD) in conjunction with flow cytometry.

Results: Cytotoxicity assessments revealed a concentration-dependent induction by TQ-Ox across the range of 1.56 to 200 µM. Notably, TQ-Ox effectively reduced the proliferation of U87 cells, with determined IC50 doses at 193.67 µM. Furthermore, a higher rate of necrosis, constituting 73.2%, was observed at 150 µM concentration ($p<0.05$).

Discussion: Our observations propose that TQ-Ox elicits dose-dependent cytotoxic and apoptotic effects on glioma cells. Eventually, it may be a candidate compound for the treatment of brain cancer and additional in vitro and in vivo research on this TQ-Ox may be required.

**Keywords**

Apoptosis, Cytotoxicity, Glioma Cell, U87, Thymoquinone-Oxime

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## Introduction

Glioblastoma represents the prevailing high-grade astrocytoma, categorized as a WHO grade IV astrocytoma. This variant constitutes 45% of all primary malignant central nervous system neoplasms and is associated with the most serious clinical prognosis [1]. This clinical inadequacy can be attributed to a multitude of factors, encompassing tumor heterogeneity, susceptibility to mutational events, heightened tumor infiltration, and augmented expression of focal adhesion kinase (FAK) protein within glioblastoma. This elevated FAK protein expression is correlated with escalated rates of both migratory and invasive behaviors [2]. Under the prevailing paradigm of treatment, which involves maximal yet safe surgical resection followed by a combination of external beam radiation and concurrent chemotherapy, the median overall survival duration for individuals confronting a fresh diagnosis of glioblastoma multiforme (GBM) merely extends to a span of 12 to 15 months [3, 4].

Thymoquinone constitutes the primary bioactive constituent within the unprocessed extract of Nigella sativa Linn seeds, and scientific investigations have indicated that TQ possesses noteworthy antineoplastic attributes [5, 6]. Numerous investigations have demonstrated the inhibitory impact of thymoquinone on the proliferation of different utilicancer cell types. These encompass breast, colon, ovarian, laryngeal, and lung malignancies, as well as myeloblastic leukemia and osteosarcoma [7]. The antineoplastic efficacy of TQ has been substantiated across various in vivo and in vitro models targeting diverse tumor types. TQ has demonstrated the ability to traverse the blood-brain barrier, prompting apoptosis in glioma and glioblastoma cells. Furthermore, TQ displays antimetastatic and anti-invasive attributes [8]. TQ exhibits preferential cytotoxicity towards glioblastoma cells when contrasted with normal human astrocytes [6]. TQ-induced tumor cell apoptosis operates through a molecular framework involving the activation of caspase 8, 9, and 3, an increase in Bax expression coupled with a decrease in Bcl2 levels, as well as the impediment of PI3K/Akt and NF-κB signaling pathways [6, 9].

Oxime compounds, due to their notable roles as acetylcholinesterase reactivators, have been under investigation for decades. They have also been explored for their potential antibacterial, antifungal, anti-inflammatory, antioxidant, and anti-cancer activities against a range of diseases [10, 11]. Oximes have been employed in formulating diverse kinase inhibitors, encompassing inhibitors of phosphatidylinositol 3-kinase (PI3K), phosphorylase kinase (PhK), and Jun N-terminal kinase (JNK). A number of these kinases serve as molecular targets for compounds exhibiting anticancer properties [11, 12].The derivatives were assessed against cancer cell lines originating from various tissues, including the colon, central nervous system, lung, breast, and ovary. All derivatives exhibited notable and promising anticancer characteristic [12, 13].

## Material and Methods

### *Thymoquinone-oxime Synthesis*

In the context of this investigation, we explored the therapeutic impact achieved through the incorporation of an oxime functional group into the TQ compound, which possesses potent anticancer attributes. In this study, we synthesized the oxime derivative of TQ, characterized by combined lipophilic and hydrophilic traits according to the principles reported previously [13]. Subsequently, we evaluated the cytotoxic and apoptotic characteristics of TQ-Ox within the U87 glioma cell line. TQ-Ox on U87 cells was examined by H-NMR, C-NMR, and elemental analysis [13].

### *Cell line treatment*

The U87 cell line was cultured within an incubator containing 5% CO2 at 37°C using Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). All reagents were sourced from Sigma-Aldrich (Seelze, Germany).

### *Cytotoxicity*

The evaluation of TQ-Ox cytotoxicity involved the use of a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay procured from Sigma-Aldrich, Seelze, Germany. The U87 cell line was seeded into a 96-well plate at a density of 8 x 103 cells/mL and incubated under 5% CO2 at 37°C with saturated humidity for a 24-hour duration. Subsequently, the medium was subjected to 1.56-200 μM concentrations of TQ-Ox for an additional 24 hours. After this incubation period, 10 μL of MTT solution (5 mg/mL) was introduced to each well and allowed to incubate at 5% CO2 and 37°C for 3 hours. Following this, 100 μL of dimethyl sulfoxide (DMSO) from Sigma-Aldrich, Seelze, Germany was added to each well and incubated at 24°C for 20 minutes. The resultant purple solution was quantified spectrophotometrically at 540 nm using a BioTek Synergy™ HTX Multimode Reader based in the USA.

### *Apoptosis*

To discern apoptotic cells, the Annexin V-Cyanine5/7-AAD Apoptosis kit (Elabscience®, E-CK-A222, USA) was employed. This approach hinges on the calcium-dependent binding of Annexin V to phosphatidylserine. Specifically, Annexin V-Cyanine5, a variant conjugated with Cyanine5, binds to phosphatidylserine on the external surface of apoptotic cell membranes, a phenomenon that can be detected through flow cytometry. In addition, the 7-aminoactinomycin D (7-AAD) dye was utilized to stain DNA within cells undergoing late apoptosis or necrosis. By simultaneously utilizing Annexin V and 7-AAD, it becomes possible to differentiate cells at various stages of apoptosis.

Cell suspensions, spanning concentrations of 25 μM, 50 μM, 100 μM, 150 μM, and 200 μM, were subjected to 7-AAD staining to facilitate the identification of apoptotic cells. The experimental protocol adhered closely to the guidelines provided by the manufacturer. Following the incubation of cell suspensions with Annexin V-Cyanine5 and 7-AAD, flow cytometry analysis (BD, FACS Canto II; Ex/Em: 488/525 nm) was employed to evaluate the apoptotic progression of glioma cells.

### *Statistical Analysis*

The obtained data were analyzed using the SPSS version 26.0 (Chicago, IL, USA). The Independent samples Kruskal-Wallis H test was used to compare more than two independent parameters. Pairwise comparisons were performed using the Dunn-Berferonni post-hoc method. All experiments were done in quadruplicate, and data were expressed as the mean (SD) of

the number of experiments. The IC50 values of TQ-Ox on cell line U87 were calculated by nonlinear regression analysis.

*Ethical Approval*

Ethical approval is not required for cell culture studies, however our study was conducted in accordance with ethical rules.

## Results

*Effect of TQ-Ox on U87 Cell Cytotoxicity by MTT*

To detect the cell cytotoxicity, U87 cells were treated with increasing concentrations of TQ-Ox (1.56-200 μM) for 24 hours. The control was with no treatment.

Cytotoxicity showed that the TQ-Ox induced in a concentration-dependent manner between 1.56 to 200 μM, see Figure 1. TQ-Ox demonstrated the capacity to attenuate the proliferation of U87 cells. No statistical difference was found when all doses were compared with the control (p>0,05). The IC50 dose of TQ-Ox wasdeterminedat193.67 Mm.

The apoptotic effect of TQ-Ox on U87 cells was determined by Annexin. U87 cells were viable at 25, 50, 100, 150 and 200 μM concentrations. Figure 2 illustrates the contrasts observed in flow cytometric outcomes when juxtaposed with control concentrations. Notably, the population of live cells

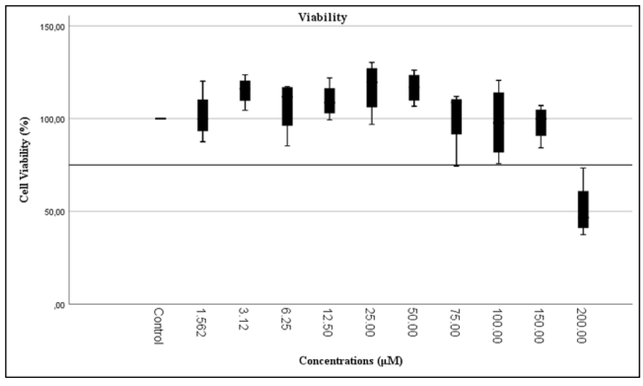


**Figure 1.** Demonstration of the capacity of TQ-Ox to reduce the proliferation of U87 cells

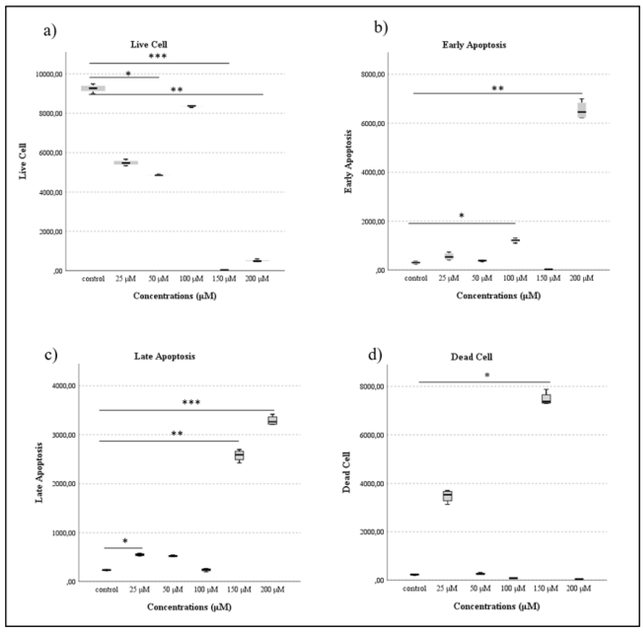


**Figure 2.** Illustration of the contrasts observed in flow cytometric outcomes when juxtaposed with control concentrations

(a) exhibited a significant decline at concentrations of 50 μM ($p<0.05$), 150 μM ($p<0.001$) and 200 μM ($p<0.01$) in comparison to the control. Additionally, early apoptosis (b) demonstrated noteworthy escalation at concentrations of 100 μM ($p<0.05$) and 200 μM ($p<0.01$) relative to the control. Late apoptosis (c) similarly showcased marked amplification at concentrations of 25 μM ($p<0.05$), 150 μM ($p<0.01$), and 200 μM ($p<0.001$) compared to the control. Lastly, the proportion of deceased cells exhibited prominence particularly at the concentration of 150 μM ($p<0.05$) when contrasted with the control.The highest necrosis was observed at 150 μMwith 73.2%.

## Discussion

Glioma stands as the prevailing manifestation of neoplasms within the central nervous system (CNS), originating from glial cells. Within the United States, the annual diagnosis rate for gliomas is reported at six cases per 100,000 individuals [14]. Several professions, environmental carcinogens, and dietary factors (including N-nitroso compounds) have been suggested to correlate with an increased risk of glioma. Yet, among these factors, the only environmental element that has been definitively linked to an elevated risk of brain tumors, including gliomas, is therapeutic X-irradiation [15]. The usual treatment involves extensive surgery, followed by chemotherapy and radiation. Being vigilant can help diagnose it early. Because the outlook is grim, it's crucial to discuss patients' care preferences soon after diagnosis, even if opting for comprehensive treatment.Progress in surgical techniques, radiotherapeutic modalities, and supplementary chemotherapy regimens has demonstrated incremental enhancements in both the survival rates and quality of life for individuals afflicted with GBM. However, the overall prognosis remains disheartening [16]. Hence, there exists a necessity to formulate novel therapeutic approaches aimed at the prevention and management of this condition. In the context of this investigation, the cytotoxic and apoptotic attributes of the oxime derivative of TQ have been explored within a human glioma cell line.

TQ's anti-inflammatory, antioxidant, and antineoplastic effects have been demonstrated in both in vitro and in vivo settings [17]. Numerous researchers have indicated that the growth-inhibitory impacts of TQ are selectively targeted towards cancerous cells [2, 6, 8, 9,13]. In a specific study, the administration of TQ led to heightened cellular levels of PTEN proteins, thereby inducing a noteworthy reduction in the phosphorylated Akt, a recognized modulator of cell survival in doxorubicin-resistant human breast cancer cells. Through flow cytometric analysis and agarose gel electrophoresis, a prominent elevation in the Sub-G1 cell populace and the emergence of distinctive DNA ladders became evident upon exposure to TQ treatment, underscoring the initiation of cellular apoptosis [18]. In an alternate study investigating the antineoplastic effects of thymoquinone on human astrocytoma cells (U87 cell line, representing a solid tumor model) and Jurkat cells (T lymphoblastic leukemia cells), TQ induced a degradation of α/β tubulin in both cancer cell varieties, a phenomenon contingent on concentration and duration. This perturbation correlated with the upregulation of the tumor suppressor p73, subsequently culminating in the induction of apoptosis [19].

Furthermore, thymoquinone prompted DNA damage, led to telomere attrition through telomerase inhibition, and elicited cellular demise within glioblastoma cells [8]. Taken together, these findings imply that concentrations of TQ which exhibit cytotoxic effects on glioblastoma cells do not impact the growth of normal human astrocytes [20]. This observation is consistent with prior studies indicating the selective nature of TQ towards cancer cells [9, 21, 22].

Over the course of numerous years, oxime compounds have been the focus of comprehensive investigations owing to their pivotal role as acetylcholinesterase reactivators and their potential utility as therapeutic agents across diverse disease contexts. The rational incorporation of an oxime moiety into an appropriate chemical structure emerges as a reasoned strategy for synthesizing cytotoxic agents. A multitude of oxime derivatives have been noted for their potential therapeutic efficacy against cancer [11]. There are studies on the treatment of glial cells with oxime compounds. In these studies, oxime-derived compounds have been shown to reduce inflammation and prevent neuronal apoptosis [23]. Due to the promising studies of oxime derivative compounds on cancer, we hypothesized that the TQ-Ox compound could affect glioma cells. Within the context of our investigation, we have indeed illustrated the cytotoxic and apoptotic impacts of TQ-Ox.

There are many studies on thymoquinone-associated cancer cells and models in the literature, but there is no relevant study investigating the efficacy of TQ-Ox to be synthesized against glioma cells to increase the efficacy of thymoquinone.In our preceding study, we assessed the effectiveness of the oxime derivative of TQ alongside established chemotherapeutic agents, Cisplatin and Taxol, against both ovarian cancer and healthy cell lines. The findings established that TQ-Ox exerts a more robust induction of cell death within the cancer cell line [13]. Furthermore, in a separate investigation, we explored the potential of TQ-Ox to hinder theproliferation of the human hepatocellular carcinoma cell line HepG2 and the healthy epithelial cell line THLE-2, employing concentrations ranging from 2.5 μM to 180 μM. Our results demonstrated a concentration-dependent pattern wherein TQ-Ox exhibited enhanced efficacy in curbing the proliferation of HepG2 cells compared to the healthy THLE-2 cells, a phenomenon that correlated with the promotion of intracellular reactive oxygen species (iROS) activity in HepG2 cells(24). Presently, our focus shifted towards investigating the impact of TQ-Ox on U87 glioma cells. The outcomes, mirroring our previous research endeavors, reveal that U87 cells treated with TQ-Ox manifest a substantial reduction in cell viability, following a concentration-dependent pattern.

## *Conclusion*

Based on the outcomes, our findings suggest that TQ-Ox exerts dose-dependent cytotoxic and apoptotic effects on glioma cells. The potent anti-proliferative effect of TQ-Ox observed in our study highlights its potential as a promising candidate for brain cancer treatment. Consequently, further in vitro and in vivo investigations into the properties of TQ-Ox may be.

***Scientific Responsibility Statement***

*The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.*

***Animal and Human Rights Statement***

*All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or compareable ethical standards.*

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***Conflict of Interest***

*The authors declare that there is no conflict of interest.*

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